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Proteome analysis of the susceptible barley - *Cochliobolus sativus* interaction

Antonious AL-Daoude and Mohammad Jawhar

Department of Molecular Biology and Biotechnology, AECS, P.O. Box 6091, Damascus, Syria
*Corresponding author e –mail: ascientific@aec.org.sy

A proteomic analysis was conducted to map up events set off during the initial stages of interaction between the fungal pathogen *Cochliobolus sativus* and the susceptible barley cultivar WI2291. Two-dimensional polyacrylamide gel electrophoresis experiments were carried to detect proteins differentially expressed under inoculated and non-inoculated conditions. Twenty-seven out of 100 protein spots were consistently observed as differential in the proteome profiles of the studied treatments. After tryptic digestion, MALDI-TOF/MS analysis and MASCOT database searching identified proteins with presumed functions relating to signal transduction, proteins involved in energy metabolism, secondary metabolism and protein synthesis. However, expression of genes involved in protection against cell damage was not detected, indicating that there may be a higher degradation of cellular components in the susceptible genotype. Proteomic results from this study indicate a complex response of susceptible barley genotype to challenge by *C. sativus* that involves simultaneous induction of proteins in the interaction system.

Key words: Barley, *Cochliobolus sativus*; proteomics; mass spectrometry

Cochliobolus sativus (Ito & Kurib.) Drechsler ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.], the cause of spot blotch (SB), is a common foliar pathogen of barley, *Hordeum vulgare*, a disease responsible for heavy crop losses (Kumar *et al.*, 2002; Mathre *et al.*, 2003). Development of SB-resistant cultivars is a high priority for many barley breeding programs worldwide. However, even susceptible hosts are not fully accessible to *C. sativus* but express different degrees of background resistance that is mainly achieved by penetration defense (Ghazvini and Tekauz 2008).

Understanding the basis of susceptibility would greatly facilitate the development of new control strategies and the identification of pathogen and host factors required for disease progression (Hückelhoven *et al.*, 2001). However, it is highly challenging to control SB in barley due to the poor understanding of the mechanisms of plant resistance and since no highly resistant barley cultivar is yet available (Kumar *et al.*, 2002). Because proteins are key components of structure and play vital roles in the cell, proteomic analysis can ideally provide direct functional information by exploring the global expression patterns of proteins in various states (Finnie & Svensson 2009; Al-daoude et al., 2013b). The primary focus of barley proteomic research to date has

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centered on detection and characterization of proteins in response to biotic and abiotic stresses (Süle *et al*., 2004; Godfrey *et al*., 2009).

Two-dimensional polyacrylamide gel electrophoresis (2-DE PAGE), coupled with matrix –assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS), is one of the most established and effective techniques to undertake proteomic analysis at the present time. These methods have been successfully applied for the identification of proteins in numerous biological systems including barley leaves and grains (Østergaard *et al.,* 2004; Datko *et al*., 2008). The advantages of this proteomic method in comparison to other methods are mainly in smaller amounts of the samples necessary and speed of the protein identification (Link *et al.* 1999; Florens and Washhburn 2006).

In spite of SB disease exerts a substantial impact on barley production in many regions of the world; limited information is available about the genetic background and regulation of interaction mechanisms in susceptible SB barley genotypes during the early phase of infection, before any visible symptoms are apparent in the tissues. Therefore, the primary objective of this research was to monitor the global response of susceptible barley leaf sheath proteins to *C. sativus* infection during the initial stages using 2-DE PAGE and MALDI-TOF/MS techniques.

MATERIALS AND METHODS

Plant material and experimental design

After an extensive screening for over ten years in the greenhouse and laboratory experiments, the Australian cv. WI2291 was proved to be the most susceptible genotype to all SB isolates available so far (Arabi and Jawhar 2004). Therefore, it was selected in this study. Plants were grown in plastic flats (60 x 40 x 8 cm) filled with sterilized peatmoss and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of 10 seedlings per genotype. A full replicate consisted of 10 pots inoculated with Pt4 isolate. Pots were placed in a growth chamber at temperatures 22 ± 1^{o}C (day) and 17 ± 1^{o}C (night) with a daylength of 12h and a relative humidity (RH) 80-90%.

Inoculum preparation

The *C. sativus* pathotype used (Pt4) was the most virulent of a collection of 117 Syrian isolates (Arabi and Jawhar 2004). It was isolated from infected barley leaves showing SB symptoms and grown separately in 9-cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and incubated for 10 days, at 21 ± 1 ^{o}C. The conidial suspension was adjusted to 2 x 10^4 conidia/mL using hemacytometer counts of conidia to provide estimates of the inoculum concentration. A surface active agent (polyoxyethylene-20-sorbitan mono-laurate) was added (100 µL/L) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surfaces.

Plant inoculation

Plants were inoculated at growth stage (GS) 11-12 (Zadoks *et al*., 1974) by uniformly spraying each plant with 25 mL of the conidial suspension with a hand-held spray bottle. After inoculation, plants were maintained in the dark at 95-100% R.H. for the first 18 h. Non-inoculated control plants sprayed with distilled water.

Protein extraction and 2-DE PAGE

Leaf sheath samples were taken at different time points (2 up to 7 days) and immediately placed on dry ice and then stored at - 80°C until protein extraction. The protein extraction method was based on that of Koller *et al.* (2002) with minor modifications. Briefly, frozen leaf sheaths were ground in a mortar with liquid nitrogen

and suspended in 10% TCA in acetone with 0.07% DTT at -20°C for 1 h, followed by centrifugation for 15 min at 35 000 g. The pellets were washed once with ice-cold acetone containing 0.07% DTT at - 20 °C for 1 h and centrifuged again for 15 min at 35 000 g. This washing step was repeated 4–5 times until the supernatant was clear. The final precipitated pellets were freeze-dried in an Advantage Freeze Dryer (VirTis, Gardiner, NY). A total of 10 mg of the dried powder was dissolved in 350 µL of buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes (pH 3–10) and 0.7% DTT. The powder dissolved in the extraction buffer was gently shaken for 1 h, and then centrifuged for 30 min. at 35 000g at room temperature. The supernatant was distributed in 100 µL aliquots and kept at -80°C until 2-D PAGE analysis. A total of 100 µg of protein, assayed by PlusOne™ 2-D Quant Kit (Amersham Biosciences, Piscataway, NJ), was loaded on to a 17-cm linear IIPG strip (Bio-Rad). The first dimensional electrophoresis (isoelectric focusing) was carried out with a Protein Isoelectric Focusing Unit (Bio-Rad) according to the manufacturer's instructions. The second dimensional electrophoresis was conducted on 12% polyacrylamide LDS (lithium-dodecyl sulphate) gel using Bio-Rad Protein II XL Gel Cell (Bio-Rad). The second electrophoresis running conditions were as follows: constant 16 mA for 30 min at 6°C followed by constant 30 mA per gel until the BPB dye reached the bottom of the gel. The 2-D gels were stained with Sypro-Ruby (Bio-Rad), and images were acquired using an FX scanner (Bio-Rad).

In-gel digestion and MALDI-TOF mass spectrometry

Target protein bands were excised from destained 12% polyacrylamdie SDS-PAGE gels manually. Protein digestion and MALDI-TOF-MS analysis were performed as described by Person *et al.* (2006). Each excised gel containing the protein of interest was placed in a protein low binding tube (Eppendorf) containing 500µL of MilliQddH_2O at 4°C for 24 h. Water was discarded and 300µL of 50mM triethyl-ammonium bicarbonate buffer (TEAB, Sigma) was added. Tubes were left on gentle shaker at room temperature (RT) for 15 min before supernatant was replaced with 50mM TEAB/50% CH3CN (acetonitrile) solution twice each for 15min at RT with gentle agitation. Supernatant was removed; 100µL of CH3CN was added to rehydrate the protein band for 5min at RT. Gel pieces were dried in a speed vacuum before they were reduced with 10mM DTT, 50mM TEAB for 1 h at 56°C and alkynated with 55mM iodoacetamide, 50mM TEAB for 45min at RT in the dark. After this treatment, each gel piece was minced and lyophilized, then swollen at 37°C overnight in 50mM TEAB containing 50ng of modified trypsin (Promega, Madison, USA). After digestion, the protein peptides were collected, and the gels were washed with 0.1% TFA in 50% CH3CN three times to collect the remaining peptides. Peptides were cleaned using C18 resin ready packed tips and diluted into freshly prepared saturated sinapinic acid dissolved in 50% acetonitrile, 0.3% trifluoroacetic acid (TFA). 2µL samples were spotted onto a stainless steel plate and spectra were collected by averaging three shots each for 200-300 lazer shots. Samples were irradiated using Bruker Microflex MALDI/TOF mass spectrometer (Bruker Daltonics, Germany) with a 377-nm nitrogen lazer, attenuated and focused on the sample target using the built-in software (Microflex package). Ions were accelerated with a deflection voltage of 30 kV and differentiated according to their m/z using a time-of-flight mass analyzer.

Database searches

Peptide masses (mass list) generated from the peptide mass fingerprint (PMFs) were used to search the NCBI database with the MASCOT search engine (Matrix Science, UK) for protein identification. The search parameters were set according to Kerim *et al.* (2003). Mascot uses a probability-based molecular weight search (Mowse) score to evaluate data obtained from tandem mass spectra. The Mowse score was reported as -10 x log (p) where p is the probability that the observed match between experimental data and the database sequence was a random event (Perkins *et al.* 1999). Mowse scores greater than 70 were considered statically significant ($p < 0.05$).

RESULTS AND DISCUSSION

In this study, a systematic shotgun proteomics approach was chosen to document the early susceptible barley response to *C. sativus* infection. It demonstrated differential gene expression in barley leaves during the early phase of infection, before any visible symptoms are apparent in the tissues. Interestingly, a difference was noticeable in the accumulation pattern of the corresponding proteins in leaves during two early stages of inoculation (Table 1). This type of early host recognition and induced biphasic transcript accumulation within hours after a fungal infection has been reported previously in barley seedlings (Gregersen *et al.*, 1997; Christensen *et al.*, 2002). Moreover, accumulation of H_2O_2 in barley leaves after infection with *C. sativus* has been reported (Schultheiss *et al.*, 2003; Al-Daoude *et al,.* 2013a).

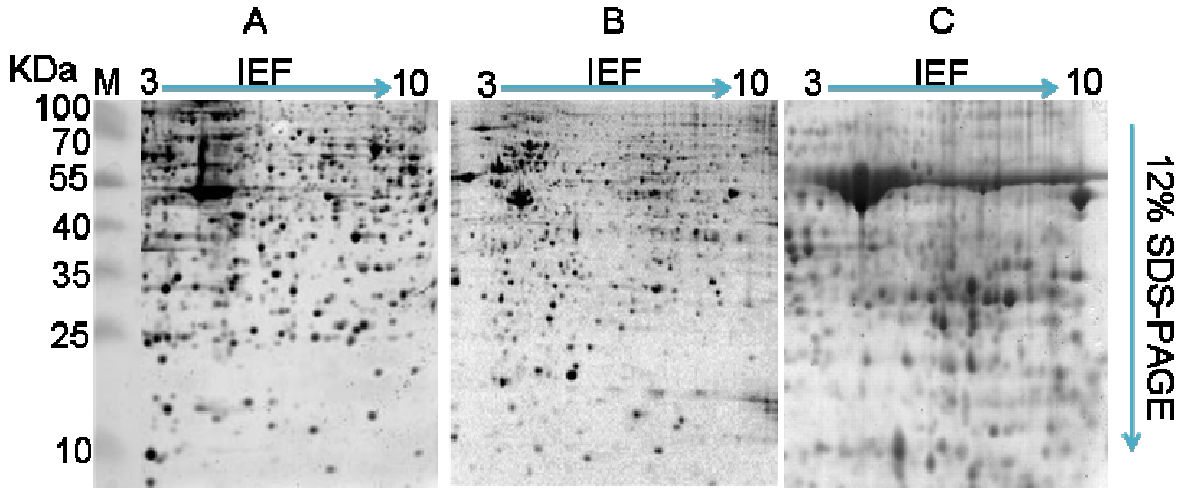

Fig. 1. 2-D gel electrophoresis of proteins extracted from unchallenged (A) and *C. sativus* challenged mature barley leaves (B &C) .

First dimension was performed on IEF with pH 3-10. In the second dimension 12% SDS-PAGE gels were used and proteins were visualized using Coomassie brilliant blue 250R.

A) Control plants (unchallenged).

B) Proteins collected 2, 3 and 4 days post inoculation).

C) Proteins collected 5, 6 and 7 days post inoculation).

M: pre-stained protein ladder (Fermentas).

Table 1. Identified proteins in the susceptible barley genotype, WI2291 at different times of inoculation with *C. sativus*

Spot no.	Protein description	Protein source	NCBI accession no.	Score	Exp. Size (kDa/Pl)	Sequence coverage
		Protein document (2, 3 and 4 days after inoculation)				
1	Unknown protein	*Arabidopsis thaliana*	gl|18399227	81	19138	25
2	Unknown protein	*Arabidopsis thaliana*	gl|21554376	75	19137	25
3	Gar1 RNA-binding region family protein	*Arabidopsis thaliana*	gl|18396707	73	21027	42
4	Putative Gar1 protein	*Arabidopsis thaliana*	gl|21536739	71	21013	42
5	Putative Gar1 protein	*Arabidopsis thaliana*	gl|6006850	80	23039	62
6	AT4g24411	*Arabidopsis thaliana*	gl|14488080	72	8316	44
7	Unknown protein	*Arabidopsis thaliana*	gl|15236953	79	19893	89
8	Luminidependens	*Arabidopsis thaliana*	gl|12655957	76	10892	1.2
9	Hypothetical protein OSI 26763	*Oryza sativa indica group*	gl|213199970	73	67365	1.4
10	RuBisco larg subunit	*Leucophyllum frutescens*	gl|131917	91	53559	32
11	Hypothetical protein	*O. sativa*	gl|101407787	89	0.1	22
12	Avirulence-resposive protein related	*Arabidopsis thaliana*	gl|42567302	89	1.22	24
		Protein document (5, 6 and 7 days after inoculation)				
13	CLP protease regulatory subunit CLPX, putative	*Arabidopsis thaliana*	gl|12322385	77	73699	0.012
14	Putative trypsin inhibitor3	*Arabidopsis thaliana*	gl|145597822	79	9745	42
15	CKL6 (casein kinase I-like 6)	*Arabidopsis thaliana*	gl|18417216	73	54163	55
16	Protein kinase ADK1-like protein	*Arabidopsis thaliana*	gl|21536851	71	53676	1.2
17	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein putative	*Arabidopsis thaliana*	gl|21536528	79	47442	74
18	CDKD1;1 (cyclin-dependent kinase D1;1); ATP binding/ kinase/protein kinase/protein serine/threonine kinase	*Arabidopsis thaliana*	gl|15219522	80	45181	1.1
19	Barley leucine zipper 1	*(Hordeum marinum subsp.gussoneanum)*	gl|213998740	87	6401	1.02
20	Calcineurin B-like protein	*Oryza sativa Japonica group*	gl|49389145	85	24358	0.25
21	Putative heat shock protein (HSP81-2)	*Arabidopsis thaliana*	gl|23397152	83	80326	0.14
22	RAC-like GTP binding protein ARAC3	*Arabidopsis thaliana*	AT4G35020	77	25431	0.21
23	Germin-like protein subfamily 1 member 14	*Arabidopsis thaliana*	NP 198727	21	23781	0.11
24	DEAD- box ATP-dependent RNA helicase 10	*Arabidopsis thaliana*	gl|28951031	77	0.19	33
25	bZIP transcriptional activator RSG, putative	*Arabidopsis thaliana*	gl|12321251	74	2	26
26	monomeric alpha-amaylase inhibitor	*Triticum turgidum*	gl|229614981	82	2.1	20
27	S-receptor kinase 13-18	*Arabidopsis lyrata*	gl|27545490	94	0.6	19

Twenty-seven out of 100 protein spots were consistently observed as differential in the proteome profiles of the inoculated and non-inoculated plants. These proteins included signal transduction, proteins involved in energy metabolism, secondary metabolism and protein synthesis (Table 1). This consisted of three sub- replications of a protein sample, two treatments of infection (inoculation vs. non-inoculation), two biological replications and one susceptible barley genotype (WI 2291). The first step involved the separation of theses protein according to their pH using IPG strips with pH values from 3-10. The second dimension was carried out using 12% acrylamide gels (Fig.1). Proteins differentially expressed between treated and nontreated plants were identified by MALDI-TOF mass spectrometry analysis and database search. Peptide mass values (data not shown) of selected proteins were used for its identification using MASCOT peptide mass fingerprint (PMF) search. PMF search identified several proteins (Table 1) with a Mowse score of ~ 85 (Fig. 2a, b.) considering that Mowse scores greater than 70 were statically significant ($P < 0.05$).

In this study, the RuBisCO large subunits were detected in barley susceptible genotype WI2291 after 2 days of inoculation, these proteins are crucial for photosynthesis (Agrios 1997). The GAR protein which is rich in glycine and arginine residues was also found after the early infection period (Table 1). After 5 days past inoculation other

enzymes related to RNA modification such as DEAD-box ATP-dependent RNA helicases 10 were observed, which catalyze the unwinding of duplex DNA in an ATP-dependent manner and thereby has have an important role in most of the basic genetic processes including replication, repair, recombination, transcription and translation (Wong and Lohman 1996).

The also detected RAC proteins in this work are involved in the regulation of susceptible plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens (Naor *et al*., 2000). Additionally, Clp proteins have been also detected after 5 days past inoculation, which are of the most critical proteolytic enzymes within photobionts (Clarke 1999), and it is now somewhat of a paradox that we currently know least about Clp protease functions in the photosynthetic organisms, where they have their most important roles. Other proteins, such as CDKD1 which is involved in the phosphorylation of proteins and regulation of cell cycle (Shimotohno *et al*., 2004), and Germin-like family of proteins catalyzing the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (Lane *et al*., 1993) were observed after 5 days of *C. sativus* inoculation (Table 1).

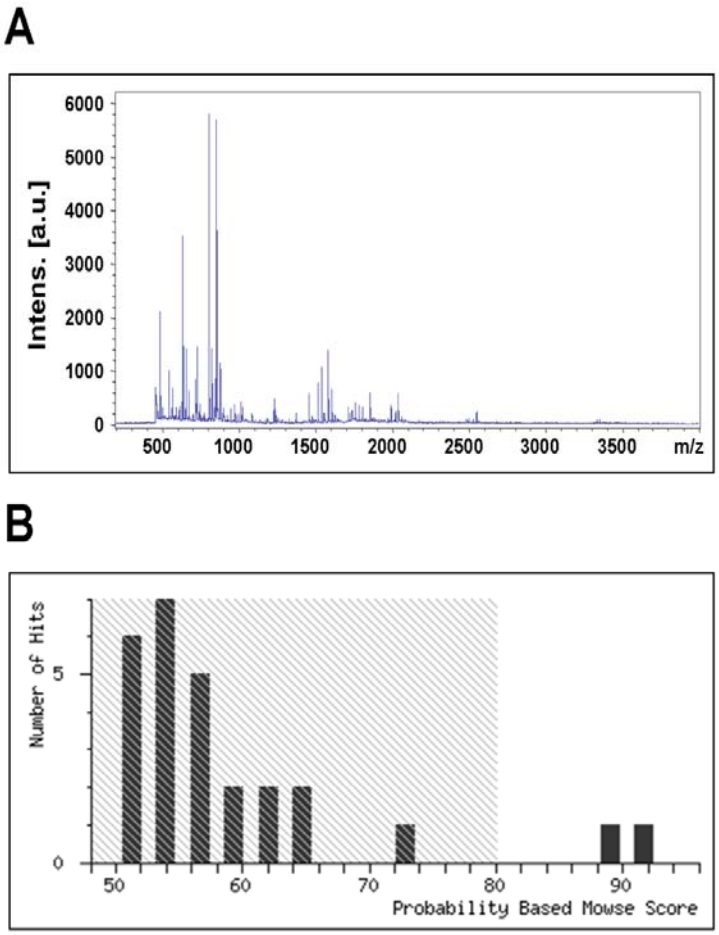

Fig. 2. Protein identification using MALDI-TOF-MS. (A) Spectra generated for a susceptible barley genotype by MALDI-TOS-MS. (B) MASCOT result identifying a significant homologue.

Additionally, heat shock proteins (HSP81-2), could also only be observed in inoculated plants after 5 days of inoculation (Table 1), these are the most well-known stress related proteins in plants which are induced in response to a number of different stresses. HSPs can play a role as chaperons which are involved in correct folding of proteins and protect them from denaturing under stress conditions (Zhu *et al.* 1995). This result was contradictory with those of Chen *et al.* (2002), in which they reported that HSP proteins were constitutively expressed and up-regulated in resistant maize lines versus susceptible lines. However, during infection, low level defense responses can be activated in susceptible plants as reported in grapevine (Fung *et al.*, 2008). Therefore, it is not surprising that well-established *C. sativus* infections involve the upregulation of genes encoding different enzymes in the beta-oxidation pathway, such as threonine protein kinases which appeared after 5 days past inoculation. Moreover, interestingly, putative trypsin inhibitor 3, a constitutively expressed antifungal protein, was detectable in the inoculated plants of the susceptible genotype WI2291 (Table 1). The true reason of up-regulation of trypsin inhibitor in our experiment remains unknown.

This proteome analysis provides a global catalogue of proteins involved in susceptible barley-*C. sativus* interaction from various response pathways. Several of the proteins detected in the current study were previously reported to be expressed by various pathogen treatments as well as various abiotic stresses, suggesting that some common response pathways exist after early stages of inoculation. However, expression of genes involved in protection against cell damage was not detected, indicating that there may be a higher degradation of cellular components in the barley susceptible genotype. Further studies are required to identify additional low-abundance, basic, hydrophobic or membrane-bound proteins associated with susceptibility to *C. sativus*.

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